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Source of ATP for hexokinase-catalyzed glucose phosphorylation in tumor cells: dependence on the rate of oxidative phosphorylation relative to that of extramitochondrial ATP generation

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Abstract

We isolated highly intact and tightly coupled mitochondria from the rat ascites hepatoma cell line AH130 by disruption of the cell membrane by nitrogen cavitation. These isolated mitochondria were found to have essentially the same functional properties as rat liver mitochondria, but unlike the latter, hexokinase (HK) was bound to their membrane. Using the tumor mitochondrial preparation, we examined the source of ATP for phosphorylation of glucose by HK under conditions in which intra- and extramitochondrial ATP-generation systems operated separately or together. Results showed that the membrane-bound HK utilized ATP derived from the most efficiently operating ATP generation system, i.e., oxidative phosphorylation. However, when the rate of extramitochondrial ATP generation was much greater than that of oxidative phosphorylation, HK used ATP from the extramitochondrial ATP-generation system.

Keywords: Tumor mitochondrion; Hexokinase; Rate of ATP synthesis; AH130 cell; Oxidative phosphorylation

Abbreviations: HK, hexokinase; GLUT1, type-1 glucose transporter; BSA, bovine serum albumin; Glc-6-P, glucose 6-phosphate; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; EM, electron microscope; TEM, transmission electron microscopy; PK, pyruvate kinase; PEP, phosphoenolpyruvate; AdK, adenylate kinase; P_i, inorganic phosphate; A_2P_5, P^1,P^5-di(adenosine-5′)-pentaphosphate; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; PCA, perchloric acid; OxP system, ATP generation system by oxidative phosphorylation; PK system, ATP generation system by exogenous PK in the presence of PEP; AdK system, ATP generation system by AdK; Glc-6-P(OxP), the amount of Glc-6-P formed in the OxP system; Glc-6-P(PK), the amount of Glc-6-P formed in the PK system; Glc-6-P(Non), the amount of Glc-6-P formed without ATP-generation system; Glc-6-P(PK + OxP), the amount of Glc-6-P formed in the presence of both PK and OxP systems.

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1. Introduction

Warburg et al. [1] first observed that tumor cells show significantly higher activity than normal cells for glucose metabolism. Subsequently, much attention has been paid to the increased activities for glucose transport and metabolism in tumor cells. Expression of the type 1 glucose transporter (GLUT1) was found to be elevated by carcinogenesis, and hence this isoform has been regarded to be mainly responsible for the enhanced glucose transport activity of transformed cells [2,3]. The mechanism of activation of expression of GLUT1 during carcinogenesis became clearer by finding of enhancer elements in its gene [4]. Besides enhancement of glucose transport, the activity of hexokinase (HK) in tumor cells is also increased to about 100-fold that of normal tissues [5–10], and of the four HK isozymes, the amount of the type II isozyme in particular was found to be increased in tumor cells [10–14]. Recently, we found by Northern blot analysis that the expression of type II HK is specifically and strongly enhanced in the rat hepatoma cell line AH130 over that in normal rat liver cells [15]. Subsequently, enhanced expression of type II HK has been observed in other tumor cell lines, such as human HepG2 [16], Morris hepatoma 3924A [17] and rat hepatoma AS-30D [18] cells. Therefore, the increased HK activity in tumor cells in general seems attributable to activation of transcription of the type II HK isozyme. To understand the molecular mechanism of the transcriptional activation of this isozyme in tumor cells, studies have been made on its gene by us [16,19,20] and others [18,21–26].

It is well established that a large amount of HK is bound to the mitochondrial outer membrane in tumor cells, as well as in energy requiring brain and skeletal muscle cells. Accordingly, the binding of HK to the mitochondrial membrane is supposed to be favorable for efficient use of ATP generated by oxidative phosphorylation for glucose phosphorylation in mitochondria. However, controversial results have been reported on the source of ATP for tumor mitochondrial HK: i.e., (1) ATP synthesized by oxidative phosphorylation [9]; (2) ATP produced by AdK [27]; and (3) ATP already present in the cytosol [28,29]. There is similar controversy over the source of ATP for HK bound to mitochondria in brain and skeletal muscle cells [30–33] and functional features of mitochondria-bound hexokinase were studied in detail [34–36].

These conflicting results may have arisen, at least in part, from differences in intactness of the mitochondria used, as suggested by Arora and Pedersen [9]. Disruption of the plasma membrane affects the intactness of mitochondria prepared from tumor cells. Several methods have been employed for disruption of plasma membranes, such as treatment with digitonin [8,9,37] or Nagarse [29,38,39], or mechanical methods such as sonication [8,27], homogenization [8,40] and cavitation [5].

In this study, we isolated mitochondria from the AH130 cell line by several procedures. We found that disruption of the cells by nitrogen cavitation was the most effective for preparation of highly intact mitochondria. Using mitochondria isolated by this procedure, we examined ATP-derived glucose phosphorylation mediated by their bound HK in various ATP-generation systems under various conditions.

2. Materials and methods

2.1. Materials

SF6847 and digitonin were purchased from Wako Pure Chemical Industries, (Osaka). BSA (fatty acid-free, A-6003) and A_2P_5 were obtained from Sigma Chemical (St. Louis), and Glc-6-P dehydrogenase and PK from Oriental Yeast (Tokyo).

2.2. Tumor cells

The AH130 rat hepatoma cell line, a gift from Dr. T. Sasaki (Taiho Pharmaceutical), was grown in ascites form by inoculating it into the abdominal cavity of male Donryu rats (4 weeks old). Seven days after inoculation, the ascites were collected and the cells were separated and promptly used for preparation of mitochondria.

2.3. Disruption of tumor cells by nitrogen cavitation

All procedures were carried out at 2–4°C unless otherwise noted. A volume of 100 ml of ascites fluid obtained from 3 or 4 rats (about $6 \cdot 10^9$ cells) was centrifuged at 180 *g* for 5 min. The precipitated cells were washed three times with medium consisting of 150 mM NaCl, 5 mM KCl and 10 mM Tris-HCl

buffer (pH 7.4). The final pellet was suspended in H-medium (210 mM mannitol, 70 mM sucrose, 1 mM EGTA, 0.5% BSA and 5 mM Hepes-KOH buffer, pH 7.2), and centrifuged at 625 *g* for 5 min. Then the cells were made into a slurry and introduced into a Kontes mini-bomb cell disruption chamber. A nitrogen pressure (20 kg/cm^2) was applied to the cell suspension for 30 min, and the suspension was recovered dropwise from the outlet knob of the chamber. Undisrupted cells were removed by centrifuging the suspension at 625 *g* for 5 min.

2.4. Preparation of mitochondria from rat liver and disrupted AH130 cells

Liver mitochondria were isolated from male Wistar rats (4–6 weeks old) essentially as described by Myers and Slater [41] in 250 mM sucrose and 2 mM Tris-HCl buffer, pH 7.4. Mitochondria from disrupted AH130 cells were isolated similarly in H-medium.

2.5. Transmission electron microscopy (TEM)

For TEM analysis, mitochondria were precipitated, fixed with 2.5% glutaraldehyde solution in 0.1 M sodium cacodylate for 12 h at 4°C and then postfixed in 1% OsO_4 for 2 h at room temperature. After dehydration in a graded ethanol series, the mitochondria were embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate and examined by TEM with a Hitachi H700H electron microscope.

For quantitative analysis of EM images, 10 sections of mitochondrial preparations were selected at random and photographed at ×10,000 magnification. The purity of isolated mitochondrial preparations was determined according to Eqn. (1).

$$\text{purity}\,(\%) = N_{\text{mito}}/N_{\text{total}} \times 100 \qquad (1)$$

where N_{mito} and N_{total} represent the number of mitochondria and total number of microorganelles in EM pictures. The areas and perimeters of mitochondria were measured with an automatic image processor analyzer (Luzex F).

2.6. Characterization of mitochondria

Mitochondria (1.75 mg protein) were suspended in medium consisting of 200 mM sucrose, 2 mM $MgCl_2$, 1 mM EDTA, 10 mM potassium phosphate buffer (pH 7.4), and 0.5 μg/ml rotenone at 25°C in a total volume of 2.5 ml, and their respiration was monitored with a Clark-type oxygen probe (Yellow Spring Instrument, model 5331) with 5 mM succinate as a respiratory substrate.

The rate of ATP synthesis by oxidative phosphorylation initiated by addition of ADP was determined at 30°C, either by measuring the pH change or by quantitation of the synthesized ATP by HPLC. For the former, mitochondria were suspended in medium consisting of 200 mM sucrose, 20 mM KCl, 3 mM $MgCl_2$, 1 mM EDTA, 5 mM succinate (plus 0.5 μg/ml rotenone) and 3 mM potassium phosphate buffer (pH 7.4) and the time-dependent pH change caused by the addition of ADP (final concentrations, 30–300 μM) was monitored continuously [42]. For the measurement of synthesized ATP by HPLC, mitochondria (1.0 mg/ml) were suspended in M-medium consisting of 220 mM mannitol, 70 mM sucrose, 2 mM Hepes, 0.5 mM EGTA, 1.6 mM $MgCl_2$, 5 mM succinate, 1 mg/ml BSA (fatty acid-free), 0.5 μg/ml rotenone and 2.5 mM potassium phosphate buffer (pH 7.4). Ten seconds after addition of ADP (final concentration, 10–200 μM), the reaction was terminated by adding ice-cold 0.3 M PCA. The mixture was incubated on ice for 30 min and then centrifuged at 15,000 rpm for 5 min. The resulting supernatant was neutralized by addition of an equal volume of saturated $KHCO_3$ solution. The adenine nucleotides in samples were separated on a Shim-pack CLC-ODS column (0.6 × 15 cm, Shimadzu) in a solution (pH 6.75) of 30 mM potassium phosphate, 15 mM tetrabutylammonium, and 19% acetonitrile at a flow rate of 1.0 ml/min and their amounts were determined from their absorbances at 260 nm.

2.7. Determination of HK activity

HK activity was determined from the amount of Glc-6-P formed either in the presence or absence of an ATP-generation system essentially by the method of Arora and Pedersen [9]. ATP was generated in three systems: by oxidative phosphorylation (OxP system), by coupling of PEP to pyruvate catalyzed by PK (PK system), and from ADP catalyzed by AdK in the inter-mitochondrial membrane space (AdK sys-

tem). HK activity in the absence of these ATP-generation systems (non-ATP-generation system) was also examined. In all cases, glucose phosphorylation was initiated by addition of ATP or ADP. In the OxP system, 0.1 mg of mitochondria were suspended in M-medium supplemented with 1.0 mM glucose in a final volume of 100 μl. Similarly, the PK system was operated by exogenous PK in the presence of 1.0 mM PEP. When necessary, ATP synthesis in each of these ATP-generation systems was inhibited, i.e., in the OxP system with 2.5 μM oligomycin and 100 μM atractyloside, in the AdK system with 40 μM A_2P_5, and in the PK system by omitting PK and PEP. After incubation of mitochondria at 30°C for 1 min, glucose phosphorylation was initiated by addition of ATP or ADP. After further incubation for 2 min, the reaction was terminated with ice-cold PCA and the solution was neutralized as described above. In experiments to determine the progress of transphosphorylation from [γ-^{32}P]ATP to Glc-6-[^{32}P]P, 370 kBq of [^{32}P]P_i was added as a tracer. The total amounts of Glc-6-P formed in these reactions were determined by a reported method [9]. An aliquot of sample containing Glc-6-P was added to medium consisting of 50 μM $NADP^+$, 0.1 U of Glc-6-P dehydrogenase and 50 mM Tris-HCl buffer (pH 8.1), and the mixture was incubated at 30°C for 30 min. The absorbance at 340 nm, or fluorescence intensity at 460 nm excited at 365 nm due to formation of NADPH coupled with oxidation of Glc-6-P was measured in a Shimadzu dual wavelength spectrophotometer, model UV-3000, or in a Hitachi fluorophotometer, model 650. This method is referred to as the NADPH method. When glucose phosphorylation was determined as Glc-6-[^{32}P]P, its amount was determined by its radioactivity after its separation by TLC as described previously [9,31]. This method is referred to as the TLC method.

3. Results

3.1. Preparation and characterization of the AH130 mitochondria

The method of disruption of plasma membranes affects the extent of damage of the mitochondrial membranes. We examined several methods of disruption of AH130 cell membranes: i.e., treatment with digitonin [8,9,37] or Nagarse [29,38,39], and nitrogen cavitation [5,43,44]. Of these procedures, the nitrogen cavitation method was found to be the most effective for isolation of AH130 cell mitochondria (data not shown). The nitrogen cavitation method was performed under various conditions essentially based on the method of Storrie and Madden for preparation of mitochondria from Chinese hamster ovary cells [44], and the procedure described in Section 2 was found to be the most suitable for isolation of highly intact mitochondria from AH130 cells.

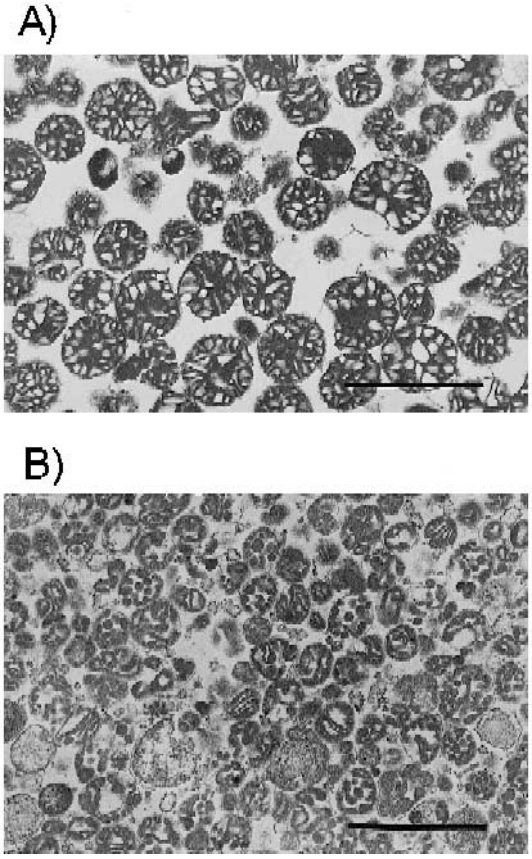

Fig. 1. TEM appearance of mitochondrial preparations. Mitochondrial fractions prepared from rat liver (A) and AH130 cells (B) were stained and photographed. Bars indicate 1.0 μm.

Fig. 1 and Table 1 show the purity and size of AH130 cell mitochondria prepared by this method in comparison with those of rat liver mitochondria. Judging from EM pictures, the 'purities' of mitochondrial fractions for rat liver and AH130 cells were 91% and 67%, respectively (cf. Eqn. (1) in Section

Table 1
Purity and size of isolated mitochondria

Source of mitochondria	Purity (%)	Area (μm^2)	Perimeter (μm)
Rat liver	91.1 ± 2.3	0.35 ± 0.03	2.12 ± 0.10
AH130 cells	67.1 ± 8.5	0.19 ± 0.02	1.54 ± 0.10

The purity of mitochondrial preparations was determined by Eqn. 1. Values are means (± S.D.) for 10 separate preparations.

2). The appearance of mitochondria of hepatoma cells was significantly different from that of normal liver mitochondria (Fig. 1), and their average size was slightly less than that of liver mitochondria (Table 1). Similar differences in size and appearance of mitochondria of other tumor cells such as Morris hepatoma cells have been reported (for a review, see [45]).

Fig. 2 shows the changes in respiratory rate (V_{ox}) of mitochondria under various conditions. With AH130 mitochondria (Fig. 2A), the slow respiration initiated by succinate (plus rotenone) increased on addition of ADP (State 3 respiratory rate, referred to as V_{ox}^{St3}). The sharp inflection of this change indicated high intactness of the mitochondrial preparation. After termination of ATP synthesis, the respiratory rate

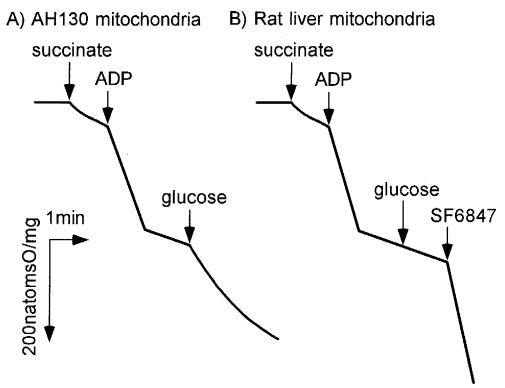

Fig. 2. Respiration of AH130 mitochondria (A) and rat liver mitochondria (B) under various conditions. Mitochondria (0.7 mg/ml) isolated from AH130 cells or rat liver were suspended in medium consisting of 200 mM sucrose, 2 mM $MgCl_2$, 1 mM EDTA, 10 mM potassium phosphate buffer (pH 7.4) and 0.5 μg/ml rotenone at 25°C in a total volume of 2.5 ml. Respiration was initiated by addition of succinate (final concentration, 5 mM). State 3 respiration was induced by 200 μM ADP. The effects of glucose and the protonophoric uncoupler SF6847 were examined at concentrations of 0.5 mM and 50 nM, respectively.

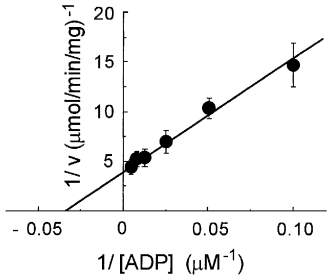

Fig. 3. Dependence of ATP synthesis rate (v) on the ADP concentration with succinate as substrate in AH130 mitochondria. The kinetic parameters of mitochondrial oxidative phosphorylation were examined with mitochondria (1.0 mg/ml) isolated from AH130 cells suspended in M-medium consisting of 220 mM mannitol, 70 mM sucrose, 2 mM Hepes, 0.5 mM EGTA, 1.6 mM $MgCl_2$, 5 mM succinate, 1 mg/ml BSA (fatty acid-free), 0.5 μg/ml rotenone and 2.5 mM potassium phosphate buffer (pH 7.4) in a total volume of 100 μl at 30°C. One minute after suspending the mitochondria, ADP was added at final concentrations of 10–200 μM [ADP], and 10 s after addition of ADP, oxidative phosphorylation was terminated by addition of ice cold PCA. After neutralization, the amounts of adenine nucleotides were determined by HPLC (see Section 2). Means (± S.E.) of at least three separate runs are shown. Essentially the same results were obtained by determination of ATP formation measured by pH change associated with ATP synthesis (data not shown).

decreased to a level similar to that before addition of ADP (State 4 respiratory rate, referred to as V_{ox}^{St4}). These features were almost the same as those with rat liver mitochondria (Fig. 2B). The index of the acceptor control ratio ($V_{ox}^{St3}/V_{ox}^{St4}$) with AH130 mitochondria was in the range of 5–6.5 with succinate as substrate, as with rat liver mitochondria. We determined the rate of ATP synthesis with succinate by measuring the amount of ATP synthesized by HPLC at various concentrations of ADP. The K_m and V_{max} values of ATP synthesis, respectively, were determined to be 31.2 μM and 271 nmol/min/mg protein with AH130 cell mitochondria (cf. Fig. 3), and 26.7 μM and 372 nmol/min/mg protein with rat liver mitochondria. The lower V_{max} value with tumor mitochondria was probably due to the lower purity of the preparation.

A significant difference between these two mitochondrial preparations was observed when glucose was added to State 4 mitochondria in the presence of ATP. An increase in the respiratory rate was observed on addition of glucose to the suspension of

tumor mitochondria due to ATP synthesis from the ADP, which would have been produced from ATP by glucose phosphorylation catalyzed by the membrane-bound HK (Fig. 2). However, the respiration did not progress linearly with time, but decreased gradually after addition of glucose, probably due to the inhibition of hexokinase activity by accumulation of Glc-6-P, which leads to decrease in ADP production. In contrast, on addition of glucose, no change was observed with rat liver mitochondria. In fact, the activity of the membrane bound HK was determined to be 786 ± 95 mU/mg protein for AH130 mitochondria, whereas that of liver mitochondria was less than 5 mU/mg protein. A similar increase in respiration on addition of glucose was reported with AS-30D cell mitochondria [9]. These results clearly showed that our mitochondrial preparation from tumor cells retained an intact membrane structure, which was favorable for efficient ATP synthesis by oxidative phosphorylation, and that HK was bound to these mitochondrial membranes. To avoid loss of intactness of the mitochondria, we used this preparation without further purification for characterization of glucose phosphorylation by membrane bound HK.

3.2. Accessibility of membrane bound HK to ATP derived from a single ATP-generation system

To determine whether the membrane-bound HK predominantly uses ATP generated in the mitochondria or extramitochondrial ATP for phosphorylation of glucose, we tested the effects of four possible sources of ATP: (1) ATP synthesized by oxidative phosphorylation (OxP system) as an intramitochondrial ATP-generation system; (2) ATP generated coupled with the conversion of exogenous PEP to pyruvate catalyzed by added PK (PK system) as an extramitochondrial ATP-generation system; (3) ATP generated by the catalysis of endogenous AdK in the intermitochondrial membrane space (AdK system); and (4) ATP added to the reaction medium in the absence of an ATP-generation system (non-ATP-generation system). When necessary, for operation of a single ATP-generation system, the OxP system was inhibited by 100 μM atractyloside and 2.5 μM oligomycin, the AdK system was inhibited by 40 μM A_2P_5, and the PK system was omitted without addition of PEP and PK. In all the systems, glucose phosphorylation was initiated by addition of ATP, at a concentration of less than the K_m value of HK, which is about 1.1 mM with type II HK [21], and the amount of ATP consumed by glucose phosphorylation was supplied by each ATP-generation system. After addition of ATP, the phosphorylation reaction was terminated by addition of PCA, and the amount of Glc-6-P produced was determined by measuring the optical absorbance or fluorescent intensity of NADPH (NADPH method, cf. Section 2).

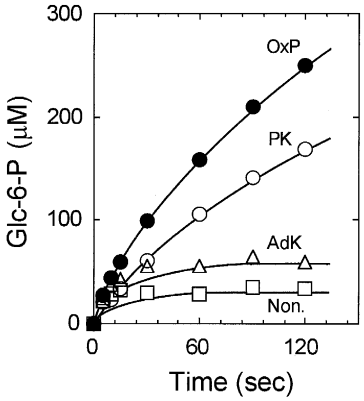

Fig. 4. Time courses of Glc-6-P formation in suspensions of AH130 cell mitochondria under various conditions. The amount of Glc-6-P produced was determined by the NADPH method in: (1) the OxP system (closed circles); (2) the PK system (open circles); (3) the AdK system (open triangles); and (4) the non-ATP-generation system (open squares). Glucose phosphorylation was initiated by adding 30 μM ATP to M-medium supplemented with 1.0 mM glucose containing 0.1 mg of AH130 mitochondria in a total volume of 100 μl. After the indicated periods of incubation at 30°C, the reaction was terminated with PCA, and the amount of Glc-6-P was determined by the NADPH method and Glc-6-P amount was shown in terms of its concentration in the reaction medium. The OxP system was operated with succinate as substrate in the presence of 40 μM A_2P_5 to inhibit the AdK system. The PK system was operated with 1 mM PEP and 10 U of PK in the presence of 100 μM atractyloside plus 2.5 μM oligomycin to inhibit the OxP system and 40 μM A_2P_5 to inhibit the AdK system. In the AdK system, ATP generation by OxP was inhibited by atractyloside and oligomycin, and PEP and PK were omitted. In the non-ATP-generation system, the OxP system and AdK system were inhibited by addition of inhibitors of these systems without addition of PEP and PK.

Fig. 4 shows the time courses of Glc-6-P production in the suspension of AH130 cell mitochondria initiated by 30 μM ATP, and the ATP consumed was supplied by a single ATP-generation system. Formation of Glc-6-P proceeded most quickly in the OxP

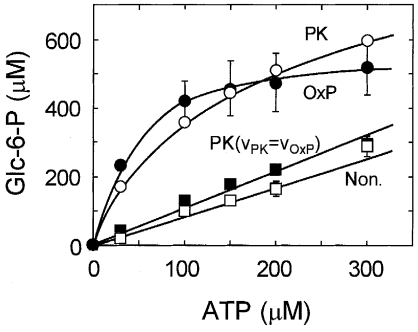

Fig. 5. Formation of Glc-6-P catalyzed by HK bound to AH130 mitochondria at various concentrations of ATP. The amount of Glc-6-P produced was determined by the NADPH method in: (1) the OxP system (closed circles); (2) the PK system with 10 U of PK (open circles); and (3) the non-ATP-generation system (open squares). Experimental conditions were essentially as for Fig. 4 except for the concentrations of added ATP and incubation period (2 min). The closed squares (PK($v_{PK} = v_{OxP}$)) represent Glc-6-P formation with a PK system in which the rate of ATP synthesis was adjusted to be the same as that in the OxP system by changing the amount of added PK (10–40 mU). Mean values ($\pm$S.E.) in at least three independent experiments are shown.

system, followed by the PK system, and in both systems it consistently increased with time for up to 120 sec. It is noteworthy that Glc-6-P formation by these two systems did not increase linearly with time. The reason for this is not clear at present. Possibly, it was the result of inhibition of hexokinase by Glc-6-P. In contrast, the phosphorylations in the AdK system and non-ATP-generation system were much lower, and attained maximum levels within 30 sec after initiation of glucose phosphorylation. The inefficient glucose phosphorylations in the latter two systems were the result of insufficient ATP productions after exhaustion of the added ATP by glucose phosphorylation. Our results were consistent with those for AS-30D cell mitochondria [9].

Fig. 5 shows the dependence of Glc-6-P formation on the ATP concentration determined 2 min after addition of ATP. As Glc-6-P formation in the AdK system was not significant (cf. Fig. 4), we examined the roles of only the OxP and PK systems in glucose phosphorylation mediated by HK, using the non-ATP-generation system as a reference. In all cases, the production of Glc-6-P increased with increase in the external ATP concentration of 30–300 μM, due to its lower concentration than the K_m value of HK. Glc-6-P formation was very high with the OxP system and PK system and much lower with the non-ATP-generation system. It is noteworthy that with the OxP system, Glc-6-P formation proceeded hyperbolically and attained nearly a maximum at about 100 μM ATP, whereas with the PK system it did not attain a maximal level even at 300 μM ATP. At the concentrations of ATP lower than 100 μM, the OxP system was more effective than the PK system. However, the difference of Glc-6-P formation in the OxP system from that in the PK system, referred to as Glc-6-P(OxP) and Glc-6-P(PK), respectively, became smaller with increase in the concentration of ATP, and Glc-6-P(PK) exceeded Glc-6-P(OxP) at more than 200 μM ATP. These changes could be associated with the difference in the K_m values of ATP synthesis in the OxP and PK systems. Namely, as the K_m of the OxP system (K_m(OxP) = 31 μM) is smaller than that of the PK system (K_m(PK) = 200 μM, [46]), glucose phosphorylation by supplementation of ATP from the OxP system attained a maximum level at a lower concentration of ATP.

These results suggested that the accessibility of HK to ATP is mainly dependent on the rate of ATP synthesis in each ATP-generation system. Therefore, the formations of Glc-6-P in the OxP and PK systems should be compared under conditions in which their rates of ATP synthesis are the same at the same ATP concentration. The values of Glc-6-P(PK) shown in Fig. 5 were those produced with a high level of PK (10 U) as used in previous reports on the accessibility of membrane bound HK to ATP [9,31,32]. At this level of PK, ATP productions by the phosphorylation of ADP formed after first turnover of glucose phosphorylation by added ATP always exceeded those by oxidative phosphorylation. It is noteworthy that formations of Glc-6-P with non-ATP-generation system, referred to as Glc-6-P(Non), were almost the same as the ATP concentrations added to initiate glucose phosphorylation. Namely, the values of Glc-6-P(Non) were 22 μM and 166 μM with 30 μM and 200 μM ATP, respectively. These values should correspond to the formation of Glc-6-P by the first turnover of glucose phosphorylation initiated by the added ATP, and the difference of either Glc-6-P(OxP) or Glc-6-P(PK) from Glc-6-P(Non) should be the Glc-6-P formed by the ATP synthesized from ADP in each ATP generation system after first turnover of glucose phosphorylation.

Therefore, we next examined glucose phosphorylation under conditions in which the rates of ATP synthesis by the OxP (v_{OxP}) and PK (v_{PK}) systems were the same. For this, we examined v_{PK} as a function of the amount of PK in the presence of 1 mM PEP at various concentrations of ADP, and determined the appropriate amount of PK to give the same ATP production as that by the OxP system ($v_{PK} = v_{OxP}$) with various concentrations of ADP. Then, we determined Glc-6-P(PK) with an amount of PK that gave the same v_{PK} as v_{OxP}. As shown by closed squares in Fig. 5 (PK($v_{PK} = v_{OxP}$)), Glc-6-P formation by the PK system was greatly decreased to a similar level to Glc-6-P(Non) under these conditions. Thus, membrane bound HK used ATP synthesized by oxidative phosphorylation preferentially, when $v_{PK} = v_{OxP}$.

3.3. Accessibility of membrane bound HK to ATP in the presence of both intra- and extramitochondrial ATP-generation systems

As described above, when only a single ATP-generation system was operational, oxidative phosphorylation was the most efficient source of ATP for Glc-6-P formation mediated by mitochondria-bound HK. However, in tumor cells, there may be competition for trapping of ADP for ATP synthesis by two or more ATP-generation systems. Thus, to know the effect of competition of ADP between these ATP generation systems, we examined the Glc-6-P synthesis in the presence of two ATP-generation systems of PK and OxP systems (PK + OxP system). The effect of the AdK system was not examined because it was essentially ineffective as reported above. To distinguish the origin of the phosphoryl group transferred to glucose from ATP, we determined the amount of Glc-6-P in two ways, as depicted in Fig. 6. First, we determined the total amount of Glc-6-P produced by the OxP and PK systems by the NADPH method (Glc-6-P(PK + OxP) in the PK + OxP system). Then under identical conditions but with $[^{32}P]P_i$ added as a tracer, we determined Glc-6-P derived from oxidative phosphorylation (Glc-6-P(OxP) in the PK + OxP system) in the form of Glc-6-$[^{32}P]$P by TLC method (see Section 2). We then calculated the amount of Glc-6-P

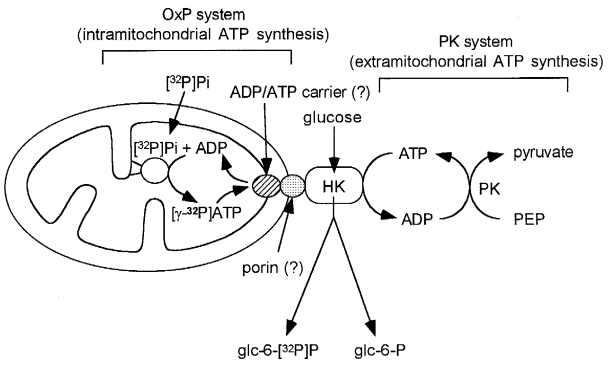

Fig. 6. Schematic representation of the experimental system to distinguish ATP from synthesized by the PK system from that synthesized by the OxP system used for glucose phosphorylation mediated by membrane-bound HK of AH130 cell mitochondria. Total amount of Glc-6-P synthesized by membrane-bound HK from ATP by the OxP and PK systems is determined by the NADPH method. Separately, under identical conditions but with $[^{32}P]P_i$ added as a tracer, the amount of Glc-6-$[^{32}P]$P formed in the OxP system is determined after its separation by TLC. The amount of Glc-6-P formed with ATP from the PK system is obtained by subtracting the amount of Glc-6-$[^{32}P]$P from the total amount of Glc-6-P. In both cases, glucose phosphorylation is initiated by addition of ADP.

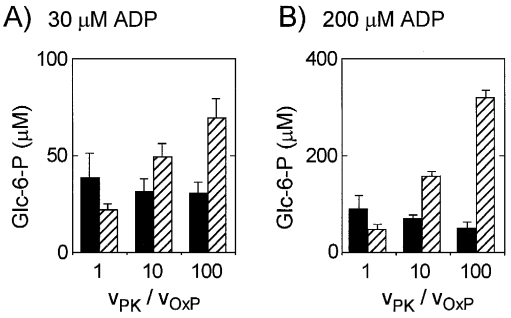

Fig. 7. Dependence of the utilization of ATP formed by the OxP and PK systems for glucose phosphorylation by HK bound to AH130 mitochondria on the rate of ATP synthesis in these systems. The formations of Glc-6-P with ATP from the OxP and PK systems were determined according to the model shown in Fig. 6. Solid columns show concentration of Glc-6-P formed with the OxP system, and hatched columns those formed with the PK system. Values are means (±S.E.) for at least three separate runs. Glc-6-P production was determined at various rates of ATP synthesis by the PK system (v_{PK}), while the rate of ATP synthesis by the OxP system (v_{OxP}) was kept constant. Glucose phosphorylation was initiated with 30 μM ADP (A) or 200 μM ADP (B). The average phosphorylation rates with the OxP system were 133 and 234 nmol ATP/min/mg protein with 30 and 200 μM ADP, respectively. Experimental conditions were essentially as for Fig. 5.

derived from the extra-mitochondrial ATP- generation system (Glc-6-P(PK) in the PK + OxP system) by subtracting Glc-6-P(OxP) from Glc-6-P(PK + OxP). In these experiments, glucose phosphorylation was determined at various v_{PK} values, while v_{OxP} was kept constant, and it was initiated by either 30 μM or 200 μM ADP instead of ATP.

Fig. 7 shows results on the Glc-6-P productions by these two ATP-generation systems when both systems were operative together. Formation of Glc-6-P were significantly dependent on v_{PK}/v_{OxP}. Under conditions in which ATP formation by oxidative phosphorylation was the same as its extramitochondrial formation ($v_{PK}/v_{OxP} = 1$), Glc-6-P(OxP) was much greater than Glc-6-P(PK) consistent with the results in Fig. 5, in which Glc-6-P synthesis initiated by ATP was examined separately in the presence of each ATP-generation system. However, Glc-6-P(PK) was greater than Glc-6-P(OxP) under the conditions in which v_{PK} was much greater than v_{OxP} with both 30 μM and 200 μM ADP. In addition, Glc-6-P(OxP) and Glc-6-P(PK) in the PK + OxP system were lower than those in the corresponding single ATP- generation system. Namely, with the same mitochondrial preparation as used in the experiments in Fig. 7, Glc-6-P(OxP) in the OxP system with 30 μM ADP was determined to be 206 μM, whereas that in the PK + OxP system was 40 μM due to competition for utilization of the added ADP for ATP synthesis between PK and OxP systems. Possibly, the effective ADP concentration for ATP synthesis by each ATP-generating system was decreased by this competition, and the ATP synthesis by the OxP system was more susceptible to this effect due to its lower K_m value. It is noteworthy that Glc-6-P(OxP) in OxP system with 30 μM ADP (= 206 μM) was very close to the corresponding value of 212 μM, which was obtained from the difference between Glc-6-P(OxP) (= 234 μM) and Glc-6-P(Non) (= 22 μM) determined in each single system with 30 μM ATP shown in Fig. 5. These results indicated that HK bound to mitochondria preferentially used ATP synthesized by oxidative phosphorylation even when the two ATP-generation systems were operational. However, when ATP production by the PK system was much greater than that by oxidative phosphorylation (v_{PK}/v_{OxP} = 10 or 100), Glc-6-P formation by the OxP system decreased slightly and that by the PK system increased significantly. As a result, under these conditions, Glc-6-P formation by the PK system became predominant.

4. Discussion

In this study, we prepared mitochondria from AH130 cells by disrupting the plasma membranes by the nitrogen cavitation method. The resulting mitochondria were well coupled and showed similar functional properties to normal rat liver mitochondria, such as essentially the same K_m value for ADP in oxidative phosphorylation (tumor mitochondria, K_m = 31 μM; rat liver mitochondria, K_m = 27 μM), although they differed from the latter in size and appearance. One of the major functional differences of tumor cell mitochondria from rat liver mitochondria was that they had appreciable membrane-bound HK, which phosphorylated glucose efficiently with an activity of 786 mU/mg protein. This value is similar to those reported for other poorly differentiated, rapidly growing tumor cell lines such as Novikoff hepatoma cells, Ehrlich ascites hepatoma cells and AS-30D hepatoma cells [7,9,10].

In tumor cells, HK should utilize ATP efficiently for glucose phosphorylation to support active glucose metabolism. HK bound to mitochondrial membranes is reported to be more effective than free HK in the cytosol for glucose phosphorylation [47]. Therefore, the membrane-bound HK of tumor mitochondria is expected to use ATP synthesized by oxidative phosphorylation preferentially for glucose phosphorylation [9]. However, the possibilities of its preferential use of ATP generated in the cytosol [28,29] or by AdK in the intermitochondrial membrane space [27] have been reported. In this study, we examined the utilizations of ATP from various sources for glucose phosphorylation catalyzed by HK bound to the outer mitochondrial membrane of AH130 cells. We examined the uses of ATP from three sources: oxidative phosphorylation (OxP system), extramitochondrial exogenous PEP and PK (PK system) and AdK (AdK system) under conditions in which the concentration of ATP or ADP was less than the K_m of HK ($\approx$ 1.1 mM, [21]). We found that glucose phosphorylation increased with increase in the concentration of ATP. Moreover ATP generated by the OxP system or PK

system was very effective for glucose phosphorylation catalyzed by HK, whereas ATP generated by the AdK system was almost as ineffective as the non-ATP-generation system. The extents of phosphorylation were associated with the efficiency of ATP supply by each system, and the inefficiencies of the AdK and non-ATP-generation systems were due to exhaustion of ATP without effective ATP generation.

As the K_m of oxidative phosphorylation in AH130 mitochondria (K_m(OxP) = 31 μM) is smaller than that of the PK system (K_m(PK) = 200 μM, [46]), ATP generation by the OxP system could be more effective than that by the PK system. In fact, even when ATP was supplied at high level by a large amount of PK, the OxP system was very effective for Glc-6-P formation at ATP concentrations of less than the 100 μM, and the effectiveness of the PK system became more significant than that of the OxP system at ATP concentrations of more than the 200 μM. However, the difference between these two ATP-generation systems was very significant when v_{PK} was adjusted to be the same as v_{OxP}. Under these conditions, the OxP system was far more effective than the PK system, indicating that it is the most favorable ATP supplier for HK activity due to its high efficiency of ATP generation.

In cells, there are several ATP-generation systems, which should compete for trapping ADP for ATP synthesis, resulting in change in the rates of glucose phosphorylation by ATP derived from each intra- and extramitochondrial ATP generation system. In fact, when the ATP generations by the OxP and PK systems operated together, the utilization of ATP derived from the extramitochondrial PK system increased greatly with increase in its ATP synthesis rate, exceeding the rate of utilization of ATP synthesized by oxidative phosphorylation, and the latter decreased with increase in ATP generation by the PK system. Therefore, utilization by HK of ATP from ATP generating systems other than the OxP system is only possible when their ATP generation rates are much greater than that by the OxP system. As the dependence of the utilization of ATP by mitochondria bound HK on the ratio of ATP formation (v_{PK}/v_{OxP}) was not affected by the concentration of ATP (Fig. 7), the observed results would be the case even in the cells in which the steady state concentration of ATP is in the range of mM.

The present results clearly showed that the accessibilities of membrane bound HK to ATP from intra- or extramitochondrial generation systems are essentially dependent on the rates of ATP synthesis by the individual ATP-generation systems. Of the intra- and extramitochondrial ATP-generation systems, oxidative phosphorylation was the most effective due to its high efficiency of ATP synthesis, overcoming the barrier to the transports of ADP, P_i and synthesized ATP across the mitochondrial membrane. The efficient use of ATP formed by oxidative phosphorylation suggests that the site of exit of ATP from mitochondria is close to the reaction site of glucose phosphorylation in HK. Porin is supposed to be a binding site of HK in the outer mitochondrial membrane [48–51]. Therefore, the ADP/ATP carrier, which mediates the transport of ADP and ATP across the inner mitochondrial membrane, is expected to be located close to porin in the outer mitochondrial membrane. Possibly, these two membrane proteins are closely associated at contact sites of the outer- and inner-mitochondrial membranes, as suggested by Adams et al. [52]. In addition, the N-terminal region of HK is reported to be responsible for its binding to the outer mitochondrial membrane [53,54]. Therefore, studies on the molecular arrangements of the ADP/ATP carrier and of porin in the membranes, and the mode of HK binding, possibly at a position close to porin, are of great importance for full understanding of the efficient utilization of ATP by HK bound to the mitochondrial membrane.

The accessibility of mitochondrial membrane-bound HK to ATP has been considered not only in tumor cells, but also in energy requiring brain and skeletal muscle cells [30–33]. As described above, our results with AH130 cell mitochondria showed that glucose phosphorylation catalyzed by HK proceeded more rapidly with increase in the concentration of ATP, and that HK preferentially utilized ATP from the most effective generation system. Due to the low K_m(OxP) value, the OxP system was far the most effective as a source of ATP for glucose phosphorylation. A similar mechanism to that in tumor cell mitochondria may be operative in other cells. The intactness of mitochondria, which was proposed to be a determinant of accessibility of HK to ATP [9], has an important influence on the efficiency of ATP synthesis by the OxP system.

We concluded that oxidative phosphorylation is the most efficient ATP source for glucose phosphorylation catalyzed by membrane-bound HK, and that another ATP-generation system is effective only when its rate of ATP generation is much greater than that by the OxP system.

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